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ORIGINAL ARTICLE

Comparison of the pathogensis of two isolates of *Besnoitia caprae* in inbred BALB/c mice

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Abstract This study was performed to evaluate the infectivity of bradyzoites of two *Besnoitia caprae* isolates, BC-1 and BC-2, to inbred BALB/c mice. Each group of inbred BALB/c mice was inoculated intraperitoneally with 1×10^3, 1×10^4, 1×10^5, 5×10^5 and 1×10^6 of one of the two isolates of *B. caprae* bradyzoites. The mice were monitored daily for a period of 40 days for survival. After death of each mice, several passages from its peritoneal washing and tissues were analyzed using ribosomal DNA-specific PCR assay. Marked differences in pathogenicity between the isolates were seen. All the inbred BALB/c mice infected with BC-2 survived but all the mice that were administered with 1×0^5, 5×10^5 and 1×10^6 BC-1 bradyzoites were died within 4–9 days post-infection (DPI). Histopathological examination of the tissues of the dead mice revealed hyperemia and necrosis with presence of mononuclear and polymorphonuclear cell infiltration in myocardium, spleen and intestines together with interstitial pneumonia and peritonitis. All inbred BALB/c mice in the 1×10^3 and 1×10^4 groups of BC-1 inoculated mice survived and they were euthanized after 40 DPI. Chronic inflammation with infiltration of mononuclear cells was evident in myocardium, spleen, alveolar septa of the lungs of most of the examined tissues with hemorrhagic enteritis in the mice infected with $1x10^6$ bradyzoites. The mice infected with different doses of BC-2 were euthanized after 40 DPI and no lesion was seen in histopathological sections of their organs. All peritoneal washings and examined tissues were PCR positive in BC-1 group. This experiment is the

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first report to show inbred BALB/c mice as a relevant model for *B. caprae* and demonstrates that this strain of inbred BALB/c mice is a suitable animal model for biological studies and examination of pathogenesis for this species of *Besnoitia*. The present findings also provide evidence for significant differences between the two isolates of *B. caprae.*

Keywords *Besnoitia caprae* · Inbred BALB/c mice · Histotopathology · Inoculation

Introduction

Parasites of the genus *Besnoitia* are classified in the subfamily toxoplasmatinae of the phylum apicomplexa (Ellis et al. 2000). Besnoitiosis is common in a wide range of domestic and wild animals (Cheema and Toofanian 1979; Bwangamoi et al. 1989; Ng'ang'a et al. 1994; Dubey et al. 2002, 2004a, 2005; Cortes et al. 2005). The genus *Besnoitia* is previously identified in nine species of animals, as intermediate hosts, including cattle, goats, sheep, equids, reindeer, caribou, opossums, rabbits, rodents, and lizards (Leighton and Gajadhar 2001; Dubey et al. 2003). However, except *B. darlingi*, *B. oryctofelisi* and *B. wallacei* that cats are their definitive hosts, the final host(s) of other *Besnoitia* species is not fully elucidated (Ng'ang'a and Kasigazi 1994; Diesing et al. 1998; Dubey et al. 2003).

The life cycle of *Besnoitia* species has not been fully elucidated. However, some species can be transmitted mechanically from one intermediated host to other susceptible intermediate hosts through tachyzoites or bradyzoites (Bigalke 1968; Ng'ang'a et al. 1994). There are several recognized and unrecognized species in the genus *Besnoitia* and morphological differences among this species are poorly defined (Dubey et al. 2003, 2005). One of the species of these protozoa is *Besnoitia caprae* that is identified as a host specific parasite in goats as intermediate hosts; while the definitive host for this protozoa is still unknown. Therefore, it will be difficult to test its actual host specificity or apply preventive measures until its natural definitive host(s) is identified. The first evidence of *Besnoitia* infection in goats was found in dried skin sections in Kenya in 1967, but the disease existed as early as 1955 (Bwangamoi 1967, 1968). However, it is stated that the *Besnoitia* of cattle and goats differ in so many of their biological properties (Njenga et al. 1993, 1995; Ng'ang'a and Kasigazi 1994), Ellis et al. (2000) reported that the *Besnoitia* cysts obtained from cattle, wildebeest and goats had identical ITS1 rDNA sequences, which questions the use of the taxon *B. caprae* as a separate species. Caprine besnoitiosis is endemic in domestic and wild goats of some of the African and Asian countries (Cheema and Toofanian 1979; Bwangamoi et al. 1989; Bwangamoi and Njenga et al. 1993; Oryan and Sadeghi 1997). The prevalence rate of caprine besnoitiosis in Fars province, southern Iran is earlier reported in the range of 12.0% (Oryan and Sadeghi (1997) to 18.9% (Oryan et al. 2008a).

Thickening, alopecia and hyperkeratosis in the skin of the infected goat occurs due to localization of *Besnoitia* cysts in the dermis and subcutaneous tissues that results in tissue necrosis and infiltration of mononuclear and eosinophils in the affected area (Dubey et al. 2004a, 2005; Njagi et al. 1998; Oryan and Sadeghi 1997; Oryan et al. 2008b). Localization of the *Besnoitia* cysts in the parenchyma of the testis and epididymis and its adverse effect on spermatogenic activity and goat production in male animals is previously reported (Kafi et al. 2007; Njenga et al. 1999b; Oryan et al. 2008a). Therefore, this parasite makes heavy economic losses by reducing the spermatogenic activity and devaluation of the skin and leather quality (Bwangamoi et al. 1989; Bwangamoi and Njenga 1993; Oryan and Azizi 2008; Njenga et al. 1999a).

Mice are commonly used as models in experiments conducted to study the biology of parasites (Lindsay et al. 1990, 1999; Cannas et al. 2003; Fux et al. 2000; Roberts and Alexander 1992; Alexander et al. 1998). Due to lack of knowledge on the transmission of this organism to laboratory animals this study was performed to present the inbred BALB/c mice as a model for biological studies and examination of pathogenesis for this species of *Besnoitia*. In addition, it is not known whether the organisms infecting the goats of different geographical areas are identical. Therefore, this experiment was designed to investigate whether the two isolates of *Besnoitia caprae* collected from two different parts of this country differed in any significant way from each other.

Materials and methods

Identification of infected goats with *Besnoitia*

Two goats, from two different geographical areas (BC-1 and BC-2), presenting manifestations of caprine besnoitiosis, consisted of scleroderma, alopecia and thickening of the skin with sand-like cysts in their conjunctiva, were subjected to skin biopsy. Skin biopsies were fixed in 10% formalin, embedded in paraffin, and 5 μm sections were processed routinely and stained with hematoxylin/eosin (H/E). *B. caprae* tissue cysts were identified by light microscopy (Fig. 1).

Isolation of *Besnoitia caprae*

Based on the Iranian Veterinary Organization legislation, the infected goats (BC-1 and BC-2) were culled and were painlessly euthanized and subcutaneous tissues of carpal and tarsal regions from these animals were collected and stored at 4°C. To remove surface contaminations, tissue pieces were separately washed in a Petri dish containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% fetal calf serum (FCS), 1% antibiotic solution (10,000 IU Penicillin and 10,000 mg Streptomycin/ml solution) and 1% amphotericin B (250 mg/ml). Subsequently, the tissue sections were cut into 1–2 mm^3

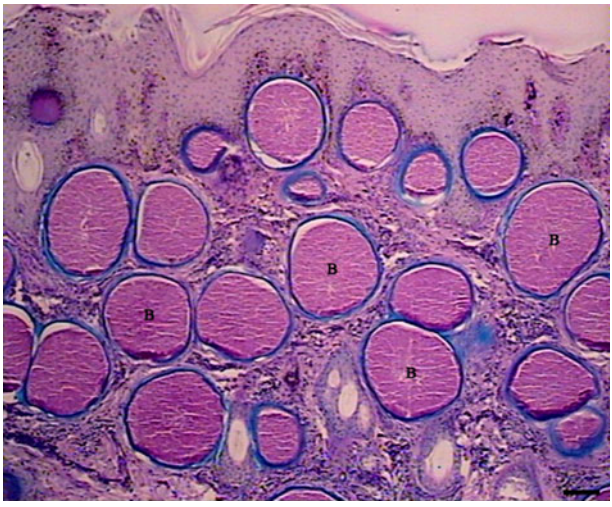

Fig. 1 *Besnoitia caprae* tissue cysts (B) in the skin of the distal part of the hind leg of an infected goat (Alcian blue/PAS. Scale bar= 300 μm)

pieces. The DMEM having liberated bradyzoites was collected and centrifuged at 770× g for 15 min at 4°C. The pellets were resuspended in DMEM and the *Besnoitia caprae* bradyzoites were counted using a neubauer chamber. These processes were undertaken under a biosafety Class II cabins and all safety measures were taken into consideration to avoid external bacterial or fungal contamination. Cryopreservation of bradyzoites of the two isolates was done at the following passage, by resuspending the bradyzoites in FCS containing 10% DMSO, and freezing and storage in liquid nitrogen.

Infection of BALB/c mice

Sixty female inbred BALB/c mice, approximately 10 weeks old, were randomly assigned into 12 groups of 5 mice. They were kept according to experimental design in individual steel cages, at 22 ± 2°C temperature and 50 ± 10% humidity and fed standard rodent proprietary mix food and fresh water. . Bradyzoites of the two isolates tested for viability with trypan blue (≥ 95% viable) and the animals of each group were inoculated intraperitoneally (IP) with a different dose of crypreserved bradyzoites of BC-1, BC-2 or a control inoculum of DMEM as indicated in Table 1. The animals were observed daily for changes in demeanour, appetite or skin appearance.

A separate experiment was conducted to investigate possibility of passage of BC-1 in mice. When each mouse infected with 1×10^5, 5×10^5 and 1×10^6 BC-1 bradyzoites died, its peritoneal washing was centrifuged at 2,500 rpm for 15 min at 4°C. The sediment was resuspended in DMEM and 5×10^5 bradyzoites were again inoculated intraperitoneally into new mice and the remainder of the sediment was freezed. Seven more passages were repeated. At each interval when the peritoneal fluid was obtained, some of the peritoneal exudates were collected and used for DNA extraction and PCR analysis.

Sample collection

The dead mice were quickly necropsied and the tissue samples were collected from the brain, lungs, heart, liver, spleen, limb's muscles and skin for PCR and histopathological evaluations. Each tissue section was cut with sterile equipment in order to prevent cross contamination. Special care was taken to avoid sampling of blood

Table 1 Experimental design

Isolate	Group	n	Inoculums
BC-1	1	5	1×10^3 bradyoites
	2	5	1×10^4 bradyoites
	3	5	1×10^5 bradyoites
	4	5	5×10^5 bradyoites
	5	5	1×10^6 bradyoites
	6	5	1×10^3 DMEM
BC-2	7	5	1×10^3 bradyoites
	8	5	1×10^4 bradyoites
	9	5	1×10^5 bradyoites
	10	5	5×10^5 bradyoites
	11	5	1×10^6 bradyoites
	12	5	1×10^3 DMEM

with the tissues by taking section blocks from the core of the organs not exposed to free fluid from the surrounding tissues. Samples were then labeled and kept in −70°C for DNA extraction and PCR analysis. The samples for histopathological evaluations were fixed in 10% neutral buffered formalin, embedded in paraffin and sections of 5 μm in thickness were stained with haematoxylin and eosin (H&E) and studied by an ordinary light microscope.

Identification of *B. caprae* by PCR

DNA extraction

One hundred microliter of peritoneal washings obtained from dead mice during serial passages (after inoculation with 1×10^5, 5×10^5 and 1×10^6 BC-1 bradyzoites) and mechanically homogenized tissue samples of lungs, heart and spleen (20 mg each) were used for genomic DNA extraction. DNA from the samples was extracted by digestion, ethanol precipitation and purification using a commercially available kit (MBST, Iran) according to the manufacture's protocol. In addition DNAs from genetically related organisms (*Neospora*- NC-1 isolate from Shiraz Razi Vaccine and Serum Research Institute-, *Sarcocystis*- from slaughterhouse and PCR positive- and *Theileria*- *T. anulata* vaccine from Shiraz Razi Vaccine and Serum Research Institute-) that are common organisms in this area were used as negative controls for checking the cross-reactivity of the primers. All the DNA samples were stored at −20°C before use.

PCR assay

Based on the high level sequence similarity between the *B. caprea* and *B. besnoiti* that were previously reported by Ellis et al. (2000), specific primers for the amplification of the ITS1-5.8 S rDNA-ITS2 regions were designed using the software Primer Premier 5.0 from the sequence of GenBank accession no. DQ227420. The primer sets used to amplify a fragment of 755 bp were Bes-F (5′-CCTCCTCACTCTGCTATCACG-3′) and Bes-R (5′-TTCCACTGGTAACGCCTCT-3′). The specificity of these primers was checked on all sequences available from the GenBank database using the Blast program (www.ncbi.nlm.nih.gov/BLAST/). The specificity of the Bes-F and Bes-R primers was also tested using negative control DNAs from genetically related organisms (*Neospora, Sarcocystis* and *Theileria*) and genomic DNA from a healthy mouse with no previous injection of bradyzoites. Primers were synthesized by CinnaGen Company (Tehran, Iran).

The following PCR conditions were applied to each assay; 50 mM KC, 10 mM Tris-HCl (pH=9.0), 1.5 mM $MgCl_2$, 200 μM dNTPs, 10 pmol of each primer, and 1 U *Taq* DNA polymerase (Fermentas, USA) per 25 μl reaction using 4 μl of DNA extracted as template. For amplification, samples were cycled in a Bio-Rad thermocycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) with an initial denaturation at 94°C for 5 min, followed by 35 cycles, denaturation at 94°C for 45 sec, annealing at 61°C for 1 min and extension at 72°C for 1 min. A final extension at 72°C for 7 min at the end of the amplification cycles was included. Sterile water and DNA genomic from non-infected mouse were used as the negative control. Presence of the PCR products was determined by electrophoresis of 7 μl of each reaction product in 2% (w/v) agarose gel with tris-borate EDTA electrophoresis buffer and visualized by staining with ethidium bromide (final concentration of 0.5 μl/ml) under UV light. Images were captured on a computer and printed.

Results

Clinical signs and mortality rate

All the mice infected with BC-2 (Groups 7–11) survived without any clinical signs during 40 DPI. As is showed in Table 2, all the mice in the groups received 1×10^5, 5×10^5 and 1×10^6 bradyzoites of BC-1 showed mortality between 4–9 days of incubation, but the two groups inoculated with 1×10^3 and 1×10^4 bradyzoites survived until 40 DPI. Infection of mice by BC-1 induced clinical symptoms comparable to the clinical signs that happen in the natural infections in goats including ruffled coat, inactivity, recumbence and anorexia (Table 3). In addition, mild hemorrhagic enteritis was evident in the mice infected with $1x10^6$ bradyzoites. At different intervals of the passages, the infected mice showed clinical signs similar to those infected in groups 3–5.

The control animals (Groups 6 and 12) showed no clinical symptoms and maintained a stable body weight throughout the experiment.

Histopathological findings

The inbred BALB/c mice inoculated with 1×10^3 and 1×10^4 of BC-1 and all mice received BC-2 were euthanized after 40 DPI. No lesion was seen in the histopathological preparations of brain, heart, lungs, liver, spleen, skin or skeletal muscles of the mice infected with BC-2 (Groups 7–11).

Histopathological examination of the tissue sections from the mice of Group 3, 4 and 5 that were infected with 1×10^5, 5×10^5 and 1×10^6 bradyzoites of BC-1, showed hyperemia, hemorrhages, interstitial pneumonia with heterophils and a few lymphocytes infiltration in the lungs. Heart sections showed areas of hemorrhages, mild necrosis and heterophils and lymphocytes infiltration (Fig. 2). Splenitis, enteritis and peritonitis with hemorrhages and mononuclear and polymorphonuclear cell infiltration were seen in these mice. The brain showed gliosis. The mice represented mild hemorrhagic enteritis in Group 5.

Chronic inflammation with infiltration of mononuclear cells was evident in brain, heart, lungs, spleen and skin and skeletal muscles of the animals of Group 1 and 2. Infiltration of macrophages and lymphocytes in the lumen of the alveoli, peribronchiolitis, development of bronchiolar associated lymphatic tissue (BALT) and interstitial pneumonia was evident in the tissue sections of these mice (Fig. 3). No cysts were found in the tissue sections. No significant lesion was found in the liver of the animals of Group 1 and 2. No lesions were

Table 2 Mortality rate of experimentally infection with BC-1

Mouse number	Dose of *B .caprae* bradyzoites				
	1×10^3	1×10^4	1×10^5	5×10^5	1×10^6
1	U40	U40	D7	D4	D4
2	U40	U40	D8	D5	D4
3	U40	U40	D8	D5	D5
4	U40	U40	D9	D6	D4
5	U40	U40	D9	D7	D5

D: died; U: euthanized; number: number of days to death or euthanasia

Table 3 Clinico-pathological changes in experimentally infected mice with BC-1

Inoculum's dose	Ruffled coat	myocarditis	Interstitial pneumonia	Hemorrhagic enteritis
1×10^3	+	+	+	−
1×10^4	+	+	+	−
1×10^5	++	++	++	−
5×10^5	+++	+++	+++	−
1×10^6	+++	+++	+++	+

+: mild; ++: moderate; +++: severe

found in the tissue sections of the brain, heart, lungs, liver, spleen, skin and skeletal muscles of the control animals (groups 6 and 12).

During the passages the infected mice showed mild necrosis with heterophils and lymphocytes infiltration in the myocardium with interstitial pneumonia.

PCR findings

In the PCR assay all of the peritoneal washings obtained from intraperitoneally passages of the animals of the fourth and fifth groups produced a single product of 755 bp similar to those of the positive control. This PCR product was not observed when the obtained DNAs from non-infected mouse were tested as the template (Fig. 4a).

Tissue samples from the mice infected with bradyzoites of BC-1 and mice from the passages were PCR positive while tissues from negative control mice (Groups 6) and genetically related parasites (*Neospora*, *Sarcocystis* and *Theileria*) were PCR negative (Fig. 4b).

Discussion

Mice models, despite their limitations, are useful for evaluation of parasites. The BALB/c mouse is used as a model of human or ovine congenital *T. gondii* infection and is suitable

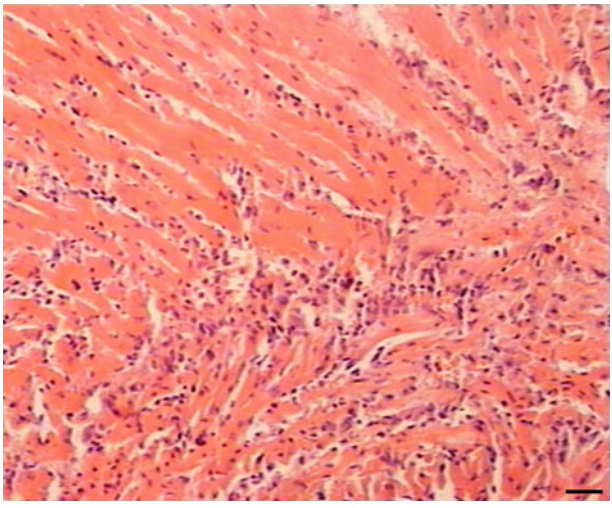

Fig. 2 Heart section from a mouse experimentally infectied with *Besnoitia caprae* bradyzoites (BC-1). Mild necrosis and infiltration of heterophils, lymphocytes and macrophages among the cardiac muscle fibers (H and E, scale bar=85 μm)

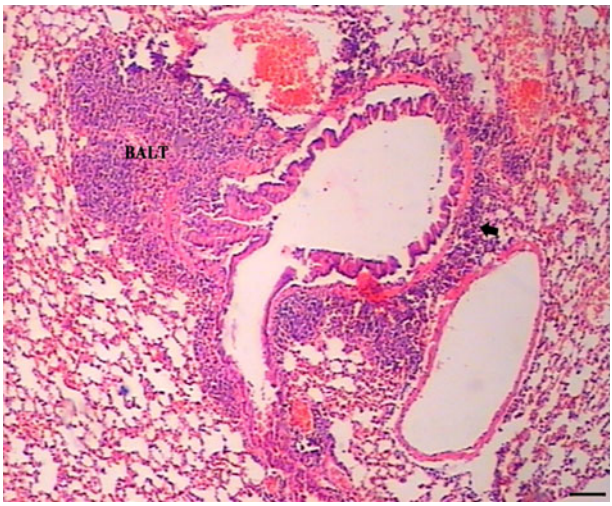

Fig. 3 Histopatholgy section from the lung of a mouse infected with 10^4 bradyzoites of *Besnoitia caprae* (BC-1). Peribronchiolitis (arrow) with bronchiolar associated lymphatic tissue (BALT) and interstitial pneumonia are prominent features of this infected lung (H and E, scale bar= 210 μm)

for testing putative vaccines (Roberts and Alexander 1992). The mice models have been used extensively in evaluating of vaccine candidates for cerebral neosporosis (Atkinson et al. 1999; Bartley et al. 2008). In addition, BALB/c and C57BL/6 mice develop clinical neosporosis and meningoencephalitis after inoculation with the parasite (Lindsay et al. 1995), so the mice particularly the BALB/c mice is showed to be susceptible to infection with some members of the apicomplexan parasites.

In the previous studies, experimental transmission of *Besnoitia caprae* was successful only in goats, but not in rabbits and mice (Njenga et al. 1993; Ng'ang'a and Kasigazi 1994). In addition, natural form of the disease due to *B. caprae* has not been reported as yet in

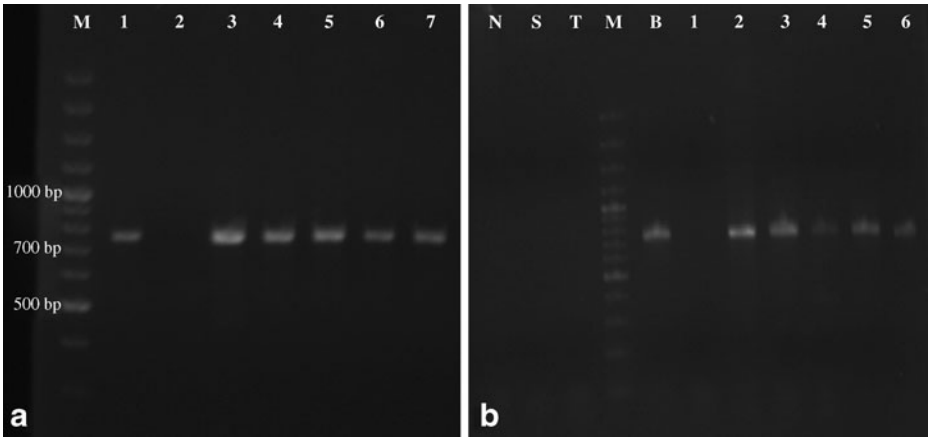

Fig. 4 **a** Electrophoretic analysis (2% agarose gel) of DNA amplified fragments from different passages of peritoneal washings in BC-1 group. M: Marker 100 bp; Lane 1: positive control; Lane 2: negative control (distilled water); Lane 3: passage 2; Lane 4: passage 3; Lane 5: passage 4; Lane 6: passage5; Lane 7: passage 6. **b** Detection of *Besnoitia caprae* in infected mice tissues with BC-1. N: *Neospora*; S: *Sarcocystis*; T: *Theileria*; M: Marker 100 bp; B: positive control (*B. caprae* DNA); Lane 1: lung from an uninfected mouse; tissues from infected mice with BC-1: Lane 2 and 3: Lung; Lane 4 and 5: Heart; Lane 6: Spleen

other animals, such as sheep, cattle, horses and donkeys that co-pastured with the infected goats (Ng'ang'a and Kasigazi 1994). *B. caprae* was therefore accepted as the specific aetiological agent for the domestic and wild goats (Cheema and Toofanian 1979; Ng'ang'a and Kasigazi 1994; Oryan and Sadeghi 1997). Oryan and Azizi (2008) reported fine structural differences between *Besnoitia* isolated from goats and *Besnoitia* of cattle in the appearance of their pellicles, micropore, orientation of the microtubules, appearance of the nucleus and the amount of lipid and amylopectin they ontain.

It is showed that the infectivity of different species of *Besnoitia* to rodents is variable (Frenkel 1977). However, it is reported that *B. jellisoni* and *B. darlingi* are infective to outbreed albino mice, *B. wallacei* did not show any infectivity to mice and *B. jellisoni* was also infective to both mice and hamsters (Frenkel 1955). It is also showed that the species of *Besnoitia* isolated from rabbits was infective to gerbils and KO mice, which are immunosuppressed mice. In another experiment, the gerbils that were inoculated with *Besnoitia* tachyzoites developed tachyzoites and tissue cysts and thus rabbit could be another candidate of animal model to maintain some of the other *Besnoitia* species (Shkap et al. 1987) but not *B. caprae*. In addition, sporulated oocysts of *B. oryctofelisi* were infective to gerbils, rabbits, outbred Swiss Webster and interferon gamma gene knockout mice and the bradyzoites of this species were infective when delivered orally to gerbils and cats. The KO mice and gerbils inoculated with *B. oryctofelisi* showed enteritis, necrosis of the cells of the lamina propria of the intestines and peritonitis (Dubey et al. 2003). It is also reported that *B. besnoiti* is able to initiate clinical besnoitiosis and cyst formation in gerbil (Shkap et al. 1987) and Gerbils were found highly susceptible to intraperitoneal infection with *B. besnoiti* (Shkap and Pipano 1993).

In another experiment, inoculation of *Besnoitia besnoiti* to laboratory animals including rabbits, hamsters, gerbils, guinea pigs and white mice, resulted to parasitemia, localization of the parasite in organs and peritoneal fluid, together with thickening, corrugation and hair loss of the skin with central nervous system disturbances without presence of *Besnoitia* cysts in different organs of these animals (Neuman and Nobel 1981). By contrary, when *Besnoitia darlingi* tachyzoites from the naturally infected opossum were propagated experimentally to mice, the cysts were formed in dermis and subcutaneous areas of these animals (Dubey et al. 2002). Our findings provide evidence for significant differences between the two isolates of *B. caprae*. However, the inbred BALB/c mice infected with high dose of BC-1 died and those infected with lower doses showed consistent pathological lesions but those infected with BC-2 remained healthy and alive and no pathological changes were evident in their different body organs. Njenga et al. (1993) and Ng'ang'a and Kasigazi (1994) showed no lesions in infected mice with *B. caprae*. Possibly the strain of *B. caprae* tested by these investigators is comparable to the BC-2 strain of the present investigation. Cortes et al. (2006) similarly reported two new isolates of *B. besnoiti;* although they stated that there is no evidence that these two isolates differ in any way with regard to morphological, structural or molecular features. However, significant biological and genetic differences between isolates of *N. caninum* are previously reported (Atkinson et al. 1999; Schock et al. 2001). Nine isolates of *N. caninum* were made from asymptomatic calves in Spain (Regidor-Cerrillo et al. 2008), and one of them (Nc-Spain-1H) failed to induce clinical signs in a BALB/c mouse, grew slowly in vitro and provided protection against fetal death in a pregnant mouse model (Rojo-Montejo et al. 2009).

Toxoplasma gondii isolates differ markedly in their virulence to outbred mice. Type-I isolates are more virulent to mice than types II and III (Dubey et al. 2004b). Cavalcante et al. (2007) represented the differences in biological performance of the two *T. gondii* isolates obtained from goats in Cear´a that G1 isolate killed all mice that had been

inoculated with 10^1, 10^2, and 10^3 tachyzoites while G2 isolate was non-lethal to mice at all concentrations tested.

In this study, BC-1 has been successfully passaged for several times by peritoneal inoculation in mice. Chai et al. (2003) reported long-term (over a year) laboratory maintenance of an isolate of *Toxoplasma gondii* in Korea (KI-1) by mouse inoculation.

Savva et al. (1990) presented that it is possible to detect *T. gondii* in body tissues and fluids by the PCR with specific oligonucleotide primers. The serial peritoneal and tissue samples PCR results confirmed presence of *B. caprae* in all samples obtained from the dead mice after inoculation with 1×10^5, 5×10^5 and 1×10^6 BC-1 bradyzoites. The *B. caprae* BC-1 used for experimental infection in mice was originally obtained from cyst material of caprine besnoitiosis. These findings indicate that the experimental transmission of bradyzoites from goats to mice is possible.

It is essential to have a better understanding of the biology and life cycle of this parasite including the possible definitive host(s) and other likely intermediate hosts, to formulate adequate control measures (Dubey et al. 2005). Developing adequate laboratory models would promote the study of the biology and epidemiology of ungulate besnoitiosis. From these findings, it could be concluded that inbred BALB/c mouse model could be suitable for biological studies and estimating the pathogenesis of this species of *Besnoitia*. Further studies should be performed in order to get a better understanding of biology of this parasite and vaccine development.

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